Virology **255,** 26–31 (1999)
Article ID viro.1998.9561, available online at http://www.idealibrary.com on IDEAL®

A Novel Epitope for the Specific Detection of Exogenous Prion Proteins in Transgenic Mice and Transfected Murine Cell Lines

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Received August 4, 1998; returned to author for revision October 6, 1998; accepted December 4, 1998

Prion diseases are closely linked to the conversion of host-encoded cellular prion protein (PrP^C) into its pathological isoform (PrP^{Sc}). PrP conversion experiments in scrapie infected tissue culture cells, transgenic mice, and cell-free systems usually require unique epitopes and corresponding monoclonal antibodies (MAbs) for the immunological discrimination of exogenously introduced and endogenous PrP compounds (e.g., MAb 3F4, which is directed to an epitope on hamster and human but not on murine PrP). In the current work, we characterize a novel MAb designated L42 that reacts to PrP of a variety of species, including cattle, sheep, goat, dog, human, cat, mink, rabbit, and guinea pig, but does not bind to mouse, hamster, and rat PrP. Therefore, MAb L42 may allow future *in vitro* conversion and transgenic studies on PrPs of the former species. The MAb L42 epitope on PrP^C includes a tyrosine residue at position 144, whereas mouse, rat, and hamster PrPs incorporate tryptophane at this site. To verify this observation, we generated PrP expression vectors coding for authentic or mutated murine PrP^Cs (i.e., codon 144 encoding tyrosine instead of tryptophan). After transfection into neuroblastoma cells, MAb L42 did not react with immunoblotted wild-type murine PrP^C, whereas L42 epitope-tagged murine PrP^C was strongly recognized. Immunoblot and fluorescence-activated cell sorting data revealed that tagged PrP^C was correctly posttranslationally processed and translocated to the cell surface.

INTRODUCTION

The accumulation of pathological prion protein (PrP^{Sc}) in neuronal tissue is a characteristic feature of all transmissible spongiform encephalopathies (TSEs). Many experimental data suggest that PrP^{Sc} represents the infectious agent itself. PrP^{Sc} develops posttranslationally from a cellular precursor protein, PrP^C, which is a membrane-bound protein and is predominantly expressed in neuronal cells of all mammalian species studied to date (Basler *et al.,* 1986). The mouse PrP gene encodes for 254 amino acids (aa) (Locht *et al.,* 1986). From the translation product, a signal peptide of 22 residues is amino-terminally cleaved off, and 23 residues at the carboxyl-terminus are substituted by a glycosylphosphatidylinositol (GPI) membrane anchor. In the Golgi apparatus, PrP^C is N-glycosylated twice at aa 180 and 196, and cysteines at aa 179 and 213 are linked by a disulphide bond (Prusiner, 1991).

The similarity in the aa sequence of donor PrP^{Sc} in comparison to PrP^C of the recipient species seems to play a crucial role in the species barrier. This has best been shown for murine and hamster scrapie using murine experimental systems (e.g., transgenic mice, transfected scrapie infected murine neuroblastoma cells, cell-free *in vitro* conversion assays) (Caughey *et al.,* 1995, 1989; Kocisko *et al.,* 1994; Scott *et al.,* 1989; Taraboulos *et al.,* 1990). These experiments were facilitated through the availability of monoclonal antibodies (MAbs), which immunologically allow the distinction of hamster and human PrPs from mouse PrP. These well characterized MAbs are directed to two epitopes on hamster PrP, one of which encompasses aa 108–111, which is characterized by two methionines instead of a leucine and a valine at the respective positions in mouse PrP (Barry *et al.,* 1986, 1986; Kascsak *et al.,* 1987; Rogers *et al.,* 1991). The prototype MAb to this epitope is MAb 3F4. The second MAb epitope is located in the vicinity of aa 138 of PrP, where mice incorporate isoleucine, whereas hamster PrP harbours methionine at this site. MAb 13A5 is the prototype MAb to this site (Barry *et al.,* 1986).

In a recent study, we introduced three novel MAb epitopes of ruminant PrP (Harmeyer *et al.,* 1998). MAbs were raised by immunization of PrP wild-type mice with synthetic peptide vaccines that were synthesized according to the aa sequence of ovine PrP. Two of the three revealed epitopes are located at the amino- and carboxyl-termini of the proteinase K-resistant core fragment of PrP^{Sc}, whereas the third epitope was resembled by a peptide spanning aa 145–163 of ovine PrP (corresponding to codons 141–159 of murine PrP). The prototype MAb to the latter domain has been designated MAb L42.

Because MAb L42 possesses unique binding features, we characterized the corresponding PrP epitope in this study. MAb L42 might provide an invaluable tool for the

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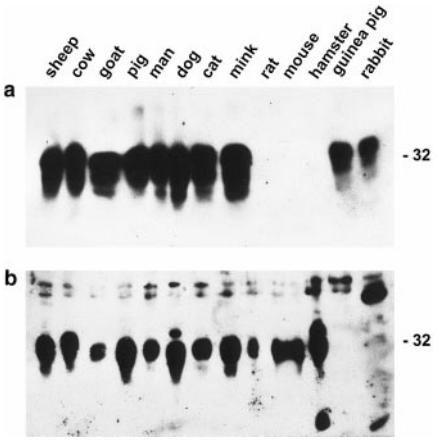

FIG. 1. Immunoblot detection of PrP^Cs of mammalian species. Immobilized metal affinity purified PrP^C preparations of sheep, cow, goat, pig, human, dog, cat, mink, rat, mouse, hamster, guinea pig, and rabbit were run on 13% polyacrylamide gels, electrotransferred onto Immobilon P membranes, and probed with (a) MAb L42, which was raised by immunization with a synthetic peptide corresponding to aa 145–163 of ovine PrP (i.e., positions 141–159 of murine PrP) and (b) polyclonal anti-peptide antibody Ra 18/4 from rabbit, which is directed to the hexapeptide repeat sequence of PrP. Although the polyclonal antibody cross-reacted with PrP^C of nearly all species, MAb L42 clearly detects all except murine, hamster, and rat PrP^Cs. Because the two blots were loaded identically, the PrP^C detection in the latter species by Ra 18/4 clearly proves the presence of antigen. Because these antibodies were raised in rabbits, they do not detect rabbit PrP. A similar immunotolerance mechanism might apply for the missing detection of PrP^C of guinea pig. The numbers on the right side indicate the approximate molecular masses as determined by marker proteins.

selection and characterization of transgenic mice expressing nonmurine PrP and might serve as a novel tag for PrP conversion experiments in scrapie infected cell lines and cell-free systems.

RESULTS

MAb L42 exhibits unique binding characteristics in respect to its species-specific detection of prion proteins. MAb L42 strongly reacts with metal affinity-purified PrP^C of a broad range of species in immunoblotting; these species include sheep, cattle, goat, pig, human, dog, cat, mink, rabbit, and guinea pig (Fig. 1). Interestingly, no reactivity is observed with PrP^C of mouse, hamster, and rat. This species-specific discrimination was equally observed when PrP^C was immunoprecipitated from crude brain tissue homogenate preparations and with immunoblotted PrP^{Sc}s of scrapie susceptible species (Harmeyer *et al.,* 1998).

MAb L42 was raised by immunization of mice with an oligopeptide covering the aa sequence 145–163 of ovine PrP^C (corresponding to codon 141–159 of murine PrP). Among the different species, three aa polymorphisms are present in this region: position 142 N ⇔ S, position 144 Y ⇔ W, and position 154 Y ⇔ H ⇔ N (aa positions calculated according to murine PrP) (Fig. 2). After comparing the reactivity of MAb L42 with the different PrP^Cs, it became clear that the polymorphism at codon 144 corresponds with the immunoblotting and immunoprecipitation results: although most species express tyrosine at this position, mouse, rat, and hamster incorporate tryptophan at this site.

Because PrP^C from guinea pig, for which the aa sequence is unknown, also bound MAb L42, we assumed that this species also carries a tyrosine residue at this PrP position. This was verified after sequencing the corresponding region of the PrP gene of guinea pig (Fig. 2).

We could further demonstrate via site-directed mutagenesis that the presence or absence of tyrosine or tryptophan at aa position 144 of murine or PrP^C of other species modulates the binding of MAb L42. To introduce a tyrosine at this site, the authentic open reading frame of murine PrP was amplified by PCR using the half-genomic vector pPrPHG as a template. This vector contains major parts of the prion gene of a s7s7 sinc genotype mouse. In a three-step PCR, a tyrosine-encoding sequence at codon 144 was introduced by using two internal mismatch primers and two external 5′ and 3′ primers, which were designed to provide unique cleavage sites. PCR products representing either the wild-type or mutated open reading frames were cloned into the vector pcDNA3.1/Zeo(+), which is particularly suitable for the overexpression of exogenous PrP^C in murine neuroblastoma (N2a) cells (Fig. 3). After sequencing of the

species	142			144										154					amino acid
	G	N	D	Y	E	D	R	Y	Y	R	E	N	M	Y	R	Y	P	N	Q
sheep	-	S	-	-	-	-	-	-	-	-	-	-	-	H	-	-	-	-	-
cow	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
goat	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pig	-	S	-	-	-	-	-	-	-	-	-	-	-	H	-	-	-	-	-
man	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
dog	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
mink	-	-	-	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
rat	-	-	-	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
mouse	-	-	-	W	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-
hamster	-	-	-	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
guinea pig	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
rabbit	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

FIG. 2. Sequence alignment of the aa 141–159 of PrP of sheep, cow, goat, pig, human, mink, rat, mouse, hamster, guinea pig, and rabbit. The aa positions were calculated according to the murine PrP sequence. Note the polymorphisms among species at positions 142, 144, and 154. The sequence for guinea pig PrP is shaded because it is reported for the first time in this report.

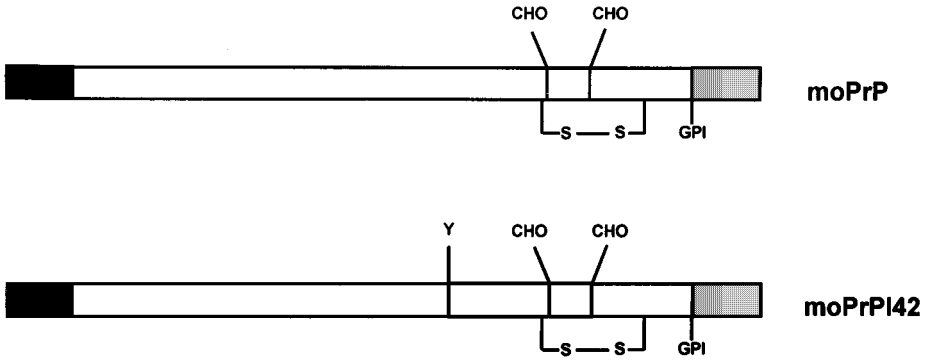

FIG. 3. Graphic illustration of the translation products in murine neuroblastoma cells transfected with the two pcDNA 3.1/Zeo(+)-based vectors pmoPrP and pmoPrPL42. The site of the aa exchange introducing tyrosine at position 144 into murine PrP is shown as well as sites of subsequent posttranslational modifications: removal of the amino-terminal signal peptide (black bars on the left), introduction of a disulfide bond, two glycosylation sites, and the cleavage site for the substitution of the carboxyl-terminus (gray bars on the right) by a GPI anchor sequence.

complete PrP open reading frames, N2a cells were stably transfected with these vector constructs coding for either wild-type or mutated murine PrP^C. Transfected cells were subsequently cloned, and individual subclones were analysed for their PrP^C expression levels by immunoblotting using a rabbit polyclonal antibody against aa 94–109 of murine PrP. Compared with normal neuroblastoma cells, N2a cells transfected with constructs coding for wild-type or L42-tagged PrP strongly overexpressed PrP. Cell lysates were also analysed by immunoblotting for the presence of the MAb L42 epitope. As predicted on the basis of the sequence data, MAb L42 strongly bound to the mutated PrP^C epitope, whereas authentic murine PrP^C was not detected with the antibody (Fig. 4). In tagged murine PrP, MAb L42 stained all three bands, which represent the nonglycosylated, monoglycosylated, and diglycosylated moieties of the protein. To confirm the posttranslational translocation of exogenous PrP to the membrane, transfected cells were also tested by fluorescence-activated cell sorting (FACS). The incubation of living neuroblastoma cells expressing either tagged or untagged PrP^C with MAb L42 revealed binding of MAb L42 only to tagged PrP^C. This shows that substantial amounts of the L42 epitope-tagged murine PrP^C were localized on the cell membrane, indicating that the post-Golgi translocation of newly synthesized protein was unimpaired by the mutation (Fig. 5).

DISCUSSION

In this study, we characterized the novel MAb L42 epitope, which is present on PrP^C of a broad range of mammalian species (e.g., sheep, cattle, goat, pig, human, dog, cat, mink, rabbit, and guinea pig) but not on murine, rat, and hamster PrP^C. In prion susceptible species, this discriminatory feature of MAb L42 also applies to the corresponding PrP^{Sc}s. The MAb L42 epitope is located in the vicinity of aa 144 of murine PrP and requires the presence of a tyrosine residue for antibody binding at this site. In contrast, a tryptophan residue at this site, as present in PrP of mouse, hamster, and rat, clearly prevents antibody binding. We verified this result with site-directed mutagenesis of murine PrP^C (i.e., by substituting tryptophan by a tyrosine-encoding codon at this site). After this mutation and the stable expression of the gene product in murine neuroblastoma cells, MAb L42 strongly reacted with mutated murine PrP^C in immunoblotted cell lysates. Recombinant L42 epitope-tagged PrP was authentically posttranslationally

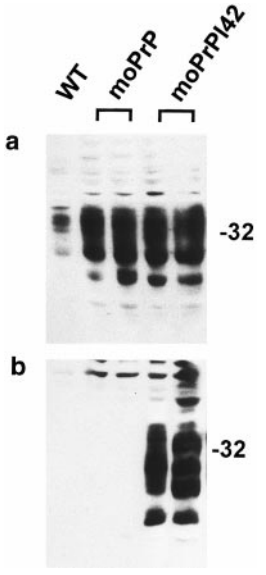

FIG. 4. Immunoblot detection of recombinant PrP, which was stably expressed in murine neuroblastoma cells. Immunoblotted cell lysates were probed using (a) a polyclonal anti-peptide antibody Ra 5/7 from rabbit to aa 94–109 of mouse PrP and (b) MAb L42. In the first lane, cell lysate from control neuroblastoma cells was applied (WT). In the two following lanes, lysates from cells that were transfected with the expression vector pmoPrP coding for authentic murine PrP were separated. In the fourth and fifth lanes, lysates from cells were probed that stably express MAb L42 epitope-tagged murine PrP. Each lane demonstrates subcloned cells of independent transfections. The number on the right side assigns the approximate molecular mass as determined by marker proteins.

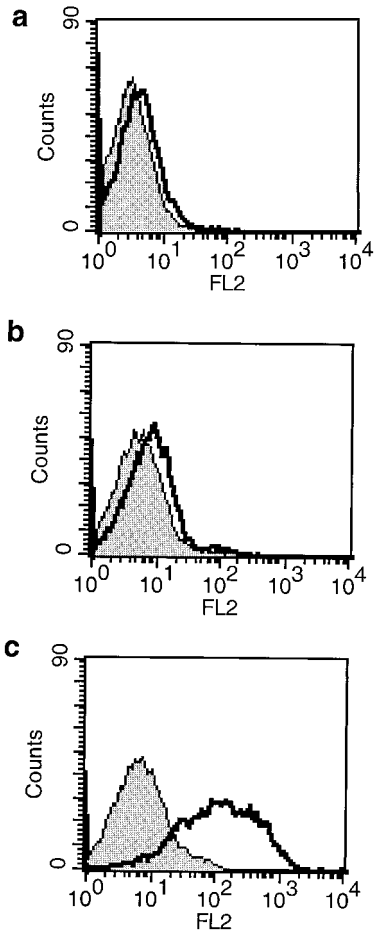

FIG. 5. FACS analysis of (a) neuroblastoma cells, (b) neuroblastoma cells transfected with pmoPrP, and (c) pmoPrPL42. Wild-type N2a cells and stably expressing cell clones were incubated with MAb L42 and a fluorescent label, and the intensity of the labeled cells was determined by FACScan (open areas). Cells incubated solely with the labeled secondary antibody served as negative controls (filled areas). Note the shift in stained cells that were transfected with the expression vector coding for MAb L42 epitope-tagged PrP (pmoPrPL42).

processed and, as FACS analysis revealed, translocated to the cell surface.

MAb L42 therefore can be very useful as a discriminatory antibody for the specific detection of exogenous PrP^C and PrP^{Sc} in mice transgenic for prion genes of a variety of TSE-susceptible species, including human, cattle, goat, sheep, and mink, and thus will be made available by the authors to scientists in the field who want to address scientific questions. Moreover, this epitope can be introduced into murine PrP by a single codon mutation encoding a tyrosine residue at aa 144 and thus may be used as a novel tag in *in vitro* PrP conversion assays such as scrapie infected murine neuroblastoma cells and cell-free systems. In the past, a variety of studies used the MAb 3F4 epitope to analyze the impact of aa sequence changes on the *in vitro* convertibility of PrP^C. However, the introduction of this tag itself partially interfered with the conversion process and substantially reduced the total PrP^{Sc} accumulation in infected cells (Priola *et al.,* 1994). Further investigations will therefore focus on whether the introduction of the L42 epitope into murine PrP^C has an impact on its convertibility in scrapie infected tissue culture cells and in transgenic mice. Another MAb to this domain, designated MAb 6H4, has been described recently (Korth *et al.,* 1994); it is directed to almost exactly the same region of PrP (aa 143–151). However, this MAb was raised in PrP-ablated mice and lacks the species specificity; it also reacts with murine and hamster PrP.

The three-dimensional structure of *Escherichia coli* expressed PrP^C has recently been determined with NMR analysis (Billeter *et al.,* 1997; Hornemann *et al.,* 1997; Riek *et al.,* 1996, 1997). According to these data, aa spanning 121–231 fold into a defined molecule that is composed of a two-stranded antiparallel β-sheet (aa 127–130, 160–163) and of three α-helices (143–153, 178–192, 199–216), whereas aa 22–120 form a long, flexible tail. According to the NMR model, the MAb L42 epitope is located within the first α-helix of murine PrP^C. In an attempt to better understand the immunogenicity and antigenicity of this domain, the predicted structure for this defined region of PrP^C was rotated in a computer simulation so the stereoscopic location of the L42 epitope became visible. Most interestingly, this epitope is located on the outbound turn of the first α-helix, with the indolyl group of tryptophan protruding almost eccentrically from the molecular surface (Fig. 6). Such an exposure is well in line with an enhanced immunogenicity and antigenicity of this domain; therefore, it is certainly feasible that the protruding side group of tryptophan at position 144 of murine PrP^C is capable of blocking the access of MAb L42 binding to its epitope. Tyrosine and tryptophan, however, are very similar in their physicochemical characteristics: both are aromatic, hydrophobic, and polar residues. Together with the structural data, this also suggests that a mutation of tryptophan into tyrosine at this site should not interfere with the overall PrP^C structure.

MATERIALS AND METHODS

Materials

Murine neuroblastoma cells (purchased from American Type Culture Collection) were maintained in minimal essential medium containing nonessential aa supplemented with 10% FCS (GIBCO) and penicillin/streptomycin in an atmosphere of 5% CO_2 at 37°C.

Polyclonal serum Ra 18/4 was raised in a rabbit against a synthetic peptide conjugate covering the aa sequence according to codons 37–53 of murine PrP (RYPGQGSPGGNRYPPQG). Ra 5/7 polyclonal antibodies were raised in a rabbit against a murine PrP peptide that corresponds to aa 94–109 (THNQWNKPSKPKTNLK). The murine MAb L42 was raised against a synthetic peptide

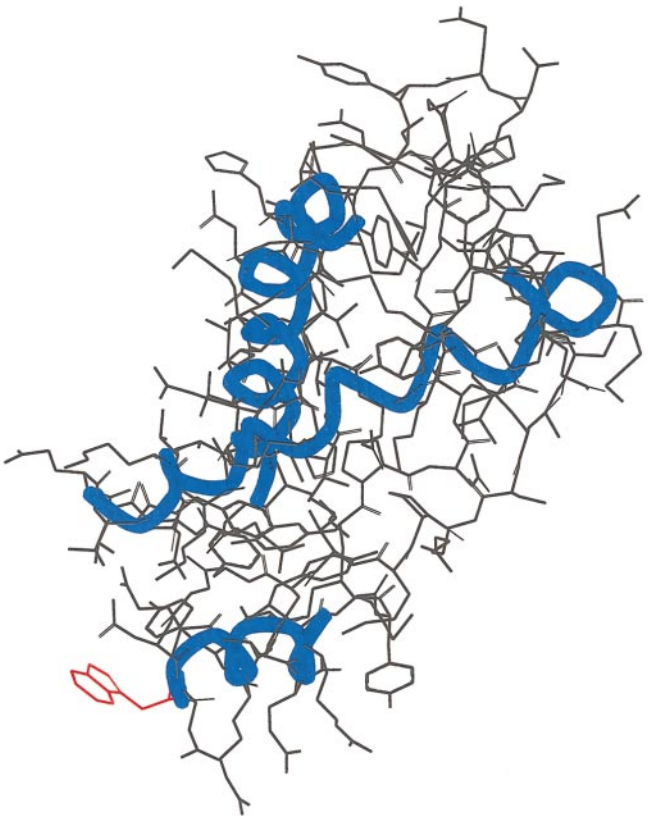

FIG. 6. Stereoscopic analysis of the MAb L42 epitope. The three-dimensional structure of murine PrP^C was based on previously published data (Billeter *et al.*, 1997; Riek *et al.*, 1996) that was retrieved from the Brookhaven Data Base and processed with the SYBYL program (Tripos Inc., St. Louis, MO). The aa backbone including side groups is shown. Illustrated by blue ribbons are the three α-helices spanning aa 143–153 (right), 178–192 (left), and 199–216 (middle helix). As depicted in red, the indolyl side group of the aa tryptophan at position 144 protrudes eccentrically from the molecule and therefore presumably blocks murine PrP^C access to the MAb L42 epitope.

that corresponds to the aa sequence 145–163 of ovine PrP (GNDYEDRYYRNMYRYPNQ).

The half-genomic murine PrP vector pPrPHG was kindly provided by Charles Weissmann (Eidgenössische Technische Hochschule, Zürich, Switzerland). The eucaryotic expression vector pcDNA3.1/Zeo(+) was purchased from InVitrogen (Carlsbad, CA).

Partial sequencing of the guinea pig PrP coding region

Brain tissue of guinea pig was used to isolate chromosomal DNA as described elsewhere (Sambrook *et al.,* 1989). The DNA served as a template to amplify a ~200-bp fragment of the PrP open reading frame with the following oligonucleotide primers: 5′-AACTACATGCTGGGGAGCG-3′ and 5′-GTGGTAATAATGACCATGTG-3′. The sequence was confirmed by sequencing using an ABI Prism 377 DNA Sequencer (Perkin–Elmer Cetus, Foster City, CA).

PrP constructs and transfection

The coding region as well as ~400 bp of the 3′ untranslated region (UTR) of wild-type mouse PrP was amplified by PCR from the plasmid pPrPHG using the following primers: 5′-TTGCCCTGCAGACTATCAGTCATCATGGCG-3′, 5′-GGCTGTTCTAGAGGGCGCCATCCC-3′. A mutant PrP open reading frame coding for tyrosine instead of tryptophan at aa 144 (numbering according to murine PrP aa sequence) was generated by PCR overlap mutagenesis using mismatch primers 5′-CCATTTTGGCAACGACTACGAGGACCGC-3′ and 5′-GCGGTCCTCGTAGTCGTTGCCAAAATG-3′ as well as the oligonucleotide primers described above. After cleavage with *Pst*I and *Xba*I, the PCR products were cloned into the expression vector pcDNA3.1/Zeo(+). In this vector, inserted sequences are under control of the human cytomegalovirus immediate-early promoter/enhancer. The fidelity of each construct was confirmed by sequencing.

Neuroblastoma cells were transfected using $CaPO_4$ (mammalian transfection kit; Stratagene, Amsterdam) according to the manufacturer's instructions. Antibiotic-resistant clones were selected in Zeozin (500 μg/ml; InVitrogen)-supplemented media and subcloned once. Experiments were carried out on single cell lines expressing each construct.

Preparation of cell lysates

Confluent cells were washed twice with PBS and lysed in 0.32 M sucrose containing 0.5% deoxycholic acid and 0.5% Nonidet P-40 for 10 min. Cell debris was pelleted and the supernatant was incubated with 10 μg/ml RNase for 30 min at 37°C.

Purification of cellular PrP

PrP^C from brain tissue of all species was extracted and purified using immobilized metal affinity chromatography as described previously (Groschup *et al.,* 1997; Pan *et al.,* 1992). Briefly, brain tissues were homogenized with an electric homogenizer in PBS containing 0.32 M sucrose, 5 mM PMSF, and 0.01 mg/ml *N*-tosyl-L-phenylalanine-chloromethylketone (TPCK). Cell debris was pelleted at 3000*g* for 30 min at 4°C, and the supernatants were supplemented with polyethylene glycol (PEG 8000; Sigma) (4% w/v). Microsomes and membrane particles were precipitated for 15 min at 4°C, pelleted at 14,000*g* for 10 min, collected, and resolubilized in PBS containing 5% Zwittergent (percent in reference to the brain mass used), 5 mM PMSF, and 0.01 mg/ml TPCK for 1 h at 4°C. The solutions were cleared of remaining microsomal debris at 100,000*g* for 1 h at 4°C, and the detergent soluble proteins were applied to Cu^{2+}-charged chelating Sepharose equilibrated with PBS containing 0.2% Zwittergent and 0.5 M sodium chloride. Bound proteins were eluted with PBS–Zwittergent containing 50 mM EDTA. The eluate was dialysed against 0.05 M Tris–

HCl, pH 8.0, for 18 h, lyophilized, and resuspended in 1/33rd of the initial volume.

SDS–PAGE and immunoblotting

For electrophoretic separation on a 13% polyacrylamide gel, 0.5× sample buffer (2% SDS, 3% sucrose, 0.001% bromphenol blue in 50 mM Tris–HCl, pH 7.4, 5% mercaptoethanol) was added to the samples. After sonication for 30 s, samples were denatured for 5 min at 95°C, electrophoresed, and finally electrotransferred to polyvinylpyrrolidone membranes (Immobilon P). Surplus binding sites were blocked with 5% nonfat dry milk solubilized in PBS supplemented with 0.1% Tween 20 for 30 min at room temperature (RT). Membranes were incubated for 2 h at RT with either polyclonal rabbit serum (Ra18/4 at a dilution of 1:500 or Ra5/7 at a dilution of 1:1500) or with monoclonal L42 hybridoma supernatant at a dilution of 1:10 in blocking solution. Membranes were washed extensively in PBS containing 0.1% Tween 20 and thereafter incubated with horseradish peroxidase-conjugated secondary antibody (Dianova, Hamburg, Germany) for 1 h. After a wash with PBS and 0.1% Tween 20, the blots were developed using the enhanced chemiluminescence reagent system (Amersham) according to the manufacturer's instructions.

FACS analysis

Cells were washed twice with PBS and detached with 250 mM sucrose, 0.25 mM EGTA, and 0.25 mM EDTA in Tris–HCl-buffered solution, pH 7.8. After pelleting, the cells were resuspended in PBS supplemented with 2% FCS and subsequently incubated with MAb L42 supernatant for 30 min at 4°C. Cells were rinsed with PBS plus 2% FCS and incubated with a 1:50 dilution of phycoerythrin-conjugated secondary antibody (Southern Biotechnology Associates) for an additional 30 min at 4°C. FACS analyses of rinsed cells were carried out using a Becton Dickinson FACStar Plus.

ACKNOWLEDGMENTS

This work was supported in parts by grants from the German "Bundesministerium für Ernährung, Landwirtschaft und Forsten," the German "Bundesministerium für Bildung, Wissenschaft und Technologie," and the EU Commission.

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